

氏 名 嶋雄一

学位（専攻分野） 博士（理学）

学位記番号 総研大乙第185号

学位授与の日付 平成20年3月19日

学位授与の要件 学位規則第6条第2項該当

学位論文題目 Ventromedial Hypothalamic Nucleus- and Pituitary
Gonadotrope-Specific Enhancer of Ad4BP/SF-1 Gene

論文審査委員 主 査 教授 長濱 嘉孝
教授 高田 慎治
教授 井口 泰泉
教授 諸橋 憲一郎（九州大学）
教授 宮田 卓樹（名古屋大学）

Ad4BP/SF-1 (Adrenal4 Binding Protein/Steroidogenic Factor-1 (NR5A1)), an orphan member of the nuclear receptor superfamily, was initially identified as a transcriptional activator of adrenal steroidogenic *P450* genes, and subsequent studies revealed that its expression is tightly regulated not only in the steroidogenic tissues such as gonad and adrenal gland, but also in the ventromedial hypothalamic nucleus (VMH), pituitary gonadotrope, and spleen. In addition to the findings above, *Ad4BP/SF-1* gene disrupted mice showed dramatic phenotypes such as adrenal and gonadal agenesis, markedly decreased gonadotropin expression, and abnormal formation of VMH. Based on these phenotypes, this factor is thought to be involved in establishment of the hypothalamic-pituitary-gonadal and hypothalamic-pituitary-adrenal axes. Since Ad4BP/SF-1 is crucial for the development of reproductive organs as well as for the endocrine regulation, extensive studies have been performed to clarify the genetic cascades regulated by Ad4BP/SF-1, and a number of target genes have been identified in each tissue. However, despite its functional significance, the molecular mechanisms underlying tissue-specific expression of Ad4BP/SF-1 are largely unknown.

In order to localize tissue-specific *cis*-regulatory regions of *Ad4BP/SF-1* gene, several cosmid clones covering the *Ad4BP/SF-1* gene locus were initially analyzed. The genomic DNA fragments in the cosmid clones were ligated to 5.8-kb *Ad4BP/SF-1* gene promoter fragment, followed by the bacterial *lacZ* gene and SV40 poly (A) signal (*Ad4BP-lacZ* cassette), and the resultant constructs were subjected to transgenic mouse assays. Among these clones, one clone termed cIA3 showed an activity to induce *lacZ* expression in the fetal adrenal gland, fetal ventral diencephalon (future VMH), and Rathke's pouch (pituitary primordium). The genomic region covered by cIA3 was analyzed further with sequentially truncated constructs, and finally he has identified the VMH-specific enhancer of *Ad4BP/SF-1* gene within the 6th intron. Interestingly, phylogenetic footprinting analysis retrospectively revealed that the VMH-specific enhancer sequence was highly conserved not only in the mammals but also in the birds and amphibians. The transgenic mouse lines harboring *Ad4BP-lacZ* cassette and the minimal enhancer fragment were then established, and expression profiles of *lacZ* were examined. From the results of these analyses, it was indicated that the enhancer has the potential to reproduce endogenous gene expression from the fetal ventromedial diencephalon to the adult VMH. In order to identify the functionally important sequences in the enhancer region, transgenic mouse analyses using the mutated or truncated enhancer fragments were performed. As the result, the enhancer was also characterized by the presence of one suppressive and two activating elements. Mutation of the former element resulted in ectopic *lacZ* reporter gene expression in an area dorsal to the intrinsic expression domain and in the ventricular zone, while mutations in the latter containing tandemly repeated ATTA motifs led to the disappearance of the reporter gene expression, suggesting the involvement of homeobox proteins in the gene regulation. Although the *trans*-acting factors capable to recognize those activating and suppressive elements have not been identified yet, electrophoretic

mobility shift assays revealed that a same factor recognizes both the two activating elements.

The pituitary gonadotrope-specific enhancer was also identified in the 6th intron of the gene by transgenic mouse assays. It was intriguing that the enhancer region was conserved only in mammals but not in the amphibians, birds, nor in the fishes although Ad4BP/SF-1 is also expressed in the pituitary of these nonmammalian species. The transgenic mouse lines in which lacZ or EGFP is driven by the minimal enhancer fragment and *Ad4BP/SF-1* gene promoter were generated. LacZ staining analyses and immunohistochemical analyses revealed that the enhancer was capable to recapitulates endogenous gene expression in the fetal Rathke's pouch later than embryonic day 13.5 to the adult pituitary gonadotrope. In addition, fluorescence-activating cell sorting and RT-PCR analyses revealed that the enhancer-driven reporter gene expression was strictly confined to the gonadotrope lineage, indicating that the enhancer function is tightly regulated only in the gonadotrope. Structurally, the enhancer included several elements completely conserved across the mammalian species, in addition to the GATA2-binding elements and EGR-1 binding element. Mutational analyses confirmed the significance of the completely conserved elements for the enhancer function, whereas mutations in the other elements showed no effects. One of the completely conserved elements was recognized by a *bicoid*-related homeoprotein, Pitx2 *in vitro*, and chromatin immunoprecipitation assays showed that the enhancer region was actually occupied by the transcriptional complex containing Pitx2 and RNA polymerase II *in vivo*. These results strongly suggested that Pitx2 regulates *Ad4BP/SF-1* gene transcription in the pituitary gonadotrope via direct interaction with the gonadotrope-specific enhancer.

During tissue differentiation, it is conceivable that certain genes encoding transcription factors act as critical components forming a gene regulatory cascade. Ad4BP/SF-1 is a component of the cascade required for differentiation of the pituitary gonadotrope and VMH. Ad4BP/SF-1 is localized upstream of a set of tissue-specific genes that include steroidogenic *Cyp* genes, and at the same time it is localized downstream of other transcription factors regulating the gene transcription. Given that activation/inactivation of the components occurs in an upstream to downstream direction along the cascade during tissue differentiation, and that Ad4BP/SF-1 is an essential transcription factor for the tissue differentiation, identification of the components that function with Ad4BP/SF-1 and regulate *Ad4BP/SF-1* gene transcription is quite important to fully understand the entire gene cascade. Identification of the enhancers to drive expressions specific for the pituitary gonadotrope and VMH is a step leading to understanding the gene cascade.

論文の審査結果の要旨

脊椎動物の内分泌機能は視床下部・脳下垂体・副腎・生殖腺軸によって制御されている。すなわち、視床下部から分泌された CRH や GnRH の刺激により脳下垂体からの ACTH や gonadotropin の産生が誘導され、これらの脳下垂体ホルモンが副腎や生殖腺における副腎皮質ホルモン及び性ステロイドの産生を促進する。一方で、分泌されたステロイドホルモンはより上位の中枢に対して負の制御を行っており、ネガティブフィードバックループを形成している。すなわち、視床下部や脳下垂体は全身の内分泌機能の制御を司る極めて重要な器官であると言える。一方、核内受容体型の転写因子である Ad4BP/SF-1 は、視床下部腹内側核 (VMH)、脳下垂体ゴナドトロピン産生細胞、副腎、生殖腺に特異的に発現しており、遺伝子破壊マウスの解析から、これら全ての組織の発生・分化過程において重要な機能を果たしていることが既に明らかにされている。このことから、本因子が制御する標的遺伝子を解析することのみならず、本因子自身に組織特異的な発現を可能にするメカニズムを知ることにより、各組織の発生の分子メカニズムを知ることが可能になると考えられる。本研究は、VMH と脳下垂体において Ad4BP/SF-1 の組織特異的な発現を制御する転写調節領域 (エンハンサー領域) を同定し、その解析から各組織における転写調節メカニズムを明らかにしたものである。

申請者は、トランスジェニックマウスを用いた解析により、VMH エンハンサーが Ad4BP/SF-1 遺伝子の第 6 イントロンに存在することを明らかにした。このエンハンサーは胎生 9.5 日齢から成獣期にいたるまで、内在性の Ad4BP/SF-1 の発現を再現する活性を有することを明らかにした。また、このエンハンサー内部に、1 つの抑制性の配列と 2 つの活性化に働く配列が存在することを明らかにした。さらに、視床下部から調整した核抽出液を用いたゲルシフトアッセイにより、2 つの活性化配列に同一の転写因子が結合することを証明した。

また、申請者は脳下垂体エンハンサーも Ad4BP/SF-1 遺伝子の第 6 イントロン、VMH エンハンサーの 1.2kb 下流に同定した。このエンハンサーは機能的には胎齢 13.5 日齢以降の Rathke 嚢から成獣の脳下垂体に至るまで、内在性の Ad4BP/SF-1 の発現を完全に再現する活性を示した。構造的には、エンハンサー内部に *bicoid* 型のホメオボックス結合配列が存在することが明らかになった。脳下垂体の発生に重要な機能を持つ Pitx2 が *bicoid* 型のホメオボックスを持つことから、申請者は Pitx2 がエンハンサーとの結合を介して Ad4BP/SF-1 の転写を制御していると仮説を立て、まず *in vitro* でこの配列が Pitx2 と結合することをゲルシフトアッセイにより示した。さらに、Pitx2 結合配列に変異を導入したトランスジェニックマウスの解析やクロマチン免疫沈降法を用いた解析により、Pitx2 が *in vivo* で確かにエンハンサーとの相互作用を介して Ad4BP/SF-1 の転写調節に関わっていることを証明した。

以上の研究成果は VMH 及び脳下垂体における Ad4BP/SF-1 遺伝子の発現調節メカニズムを初めて明らかにしたものであり、学位の取得に値すると審査員全員が判断した。